

UNCLASSIFIED

N00014-85-K-0004

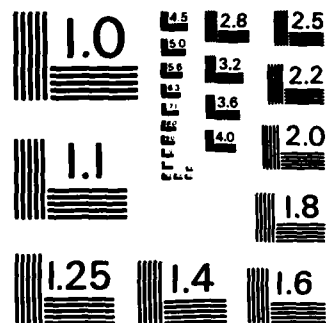
NL

F/G 6/1

END

FILMED

NTUC



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS - 1963 - A

AD-A160 704

1

2

DNA and protein studies of HLA class II molecules: their relationship to T
cell recognition

1985

Miriam Segall, J. Scott Cairns, Carol A. Dahl, Julie M. Curtsinger, Scott
Freeman, Peter J. Nelson, Oded Cohen, Shaokke Wu, Janice N. Nicklas, Harriet
J. Noreen, Kristin M. Linner, Thomas L. Saunders, Stephana A. Choong, Nobuo
Ohta, Nancy L. Reinsmoen, Barbara J. Alter and Fritz H. Bach.

Immunobiology Research Center

and

Departments of Laboratory

Medicine/Pathology and Surgery

University of Minnesota

Minneapolis, MN 55455

DTIC
ELECTE
OCT 11 1985
S D
E

THIS FILE COPY

This is paper #415 from the Immunobiology Research Center. This work was
supported in part by NIH grants AM 31342, AI 17687, AI 18326, AM 13083 and AI
19007, by the National Multiple Sclerosis Society grant #RG 1505-A-1, and a
contract from the Office of Naval Research ONR ^{N00014} ~~N00014~~-85-K-0004.

This document has been approved
for public release and sale; its
distribution is unlimited.

85 10 08 094

The HLA-D region, encoding class II antigens, was first defined based on studies of mixed leukocyte culture (MLC) (Bain, et al. 1964; Bach and Hirschhorn, 1964). In our own studies with colleagues (Amos and Bach, 1968; Bach, et al. 1969), we were able to suggest that a genetic region encoding molecules that stimulate proliferation in MLC may be linked to, but separate from, the HLA-A and -B loci, i.e. a region such as HLA-D; definitive studies in this regard (Yunis and Amos, 1971) established the existence of the HLA-D region.

Great advances have been made in our understanding of this region in recent years utilizing both the tools of molecular genetics and studies of proteins encoded by HLA-D genes. A critical bridge that has not yet been completed concerns the relationship of class II genes and protein products to determinants recognized by T lymphocytes, i.e. the cells responding in MLC. Although the emphasis in this review will be on studies of proteins and of DNA, an introduction to this article also includes our current state of knowledge regarding definition of class II products with T lymphocytes. We accept the invitation of the editor primarily to summarize our own work without reviewing the literature in the field; the inclusion in this volume of several articles on the same topic allows a measure of comfort with such an approach.

Genomic Structure of the HLA-D Region and the Protein Products

As reviewed in detail elsewhere (Bach, 1985, Kaufman et al., 1984), there are three families of genes, DR, DQ and DP, in the HLA-D region. The DR family includes one α gene and three β genes; the DQ and DP regions each include two α genes and two β genes. How many of these genes are expressed for each family is uncertain; however, it would appear that at least two and perhaps three different β genes of DR are expressed. There have been



Dist	Avail and Special
AI	

suggestions that two separate products of both DQ and DP can be expressed, although evidence in this regard is sparse.

Proteins encoded by class II genes are expressed as $\alpha\beta$ heterodimers. Beta chains of DR presumably associate with the protein product of the DR α gene, with intra-family $\alpha\beta$ associations also taking place for DQ and DP. To what extent an α chain of one family can associate with β chains of a different family is not established.

Class II Phenotyping with Alloantisera and Homozygous Typing Cells (HTCs)

The relationship of HLA to T lymphocyte reactivity has been established by demonstration of the requirement for determinants on HLA molecules for restricted recognition. However, the relative ease of obtaining alloantisera, as well as the simplicity of the techniques utilizing such reagents, has led primarily to serological definition of determinants (which we shall refer to as Ia determinants) associated with both DR and DQ. DR family-associated Ia determinants can be divided into two series based on their population distributions; recent evidence potentially relates them to different $\alpha\beta$ dimers of DR. The DR1 thru DRw14 specificities are thought to be associated with one of the DR dimers, arbitrarily $\alpha\beta_1$, whereas as the DRw52 and DRw53 (previously referred to as MT2 and MT3) specificities are thought to be related to an $\alpha\beta_2$ dimer (Hurley et al., 1984). Although the associations with $\alpha\beta_1$ have been examined for only certain of the DR1 thru DRw14 antigens, and there should therefore be some hesitation in accepting these assignments too firmly, we shall use this concept in the article.

Three serologically defined antigens are commonly recognized for the DQ family: DQw1, DQw2 and DQw3. Very little information is available regarding which DQ α and β chains are expressed. These antigens are, thus, simply referred to as encoded by alleles of DQ.

Determinants recognized by T lymphocytes as associated with HLA class II products have been defined primarily with homozygous typing cells (HTCs). Nineteen specificities, designated Dw1 to Dw19, are known. Specificities defined with HTCs are referred to as Dw (they are still ill-defined and thus referred to as "workshop" specificities); the term LD is used for individual determinants recognized by T lymphocytes. An LD epitope is presumably most often associated with a single molecular product. A Dw specificity is defined by the composite response of T lymphocytes to LD epitopes present on all products of HLA-D and thus represents a "haplotype designation".

Definition of the HLA-Dw1 through Dw19 specificities is based on proliferative responses of T lymphocytes in a primary MLC to stimulating cells that are putatively homozygous, at least for the DR and DQ products (14). HTCs are chosen as typing reagents if i) the cells do not stimulate a significant response in the appropriate combinations within the family from which they were derived, ii) they do not stimulate (or are weak in stimulating) cells of other HTCs used to "define" the same Dw specificity, and iii) they can be used successfully to "type" an unrelated panel, i.e. to distinguish between unrelated individuals whose cells respond positively in MLC to the HTC and those who show no response or a weak response.

HTC testing has been most useful in defining the series of Dw specificities which represent clusters of antigenic determinants associated with the various class II products. (Class I antigens can, when disparate, also stimulate T lymphocytes to proliferate, albeit weakly). The response to an HTC represents the aggregate reactions of clones that can recognize determinants associated chiefly with DR, DQ and DP. It depends not only on the number of determinants carried by the HTC which the responding cell does not have but also on the "strength" of those determinants, i.e. the frequency of clones that

respond to those disparate determinants and the extent of proliferation by cells of those clones.

The ability to define Dw specificities with HTC's, despite the ability of all HLA products to stimulate T cell proliferation, rests to a great extent on three factors. First, the DR product(s) may stimulate most of the proliferating cells in an MLC (Bach et al., 1983), although there is only a little evidence for this. Second, loose criteria have been used to designate a typing response; more stringent criteria, such as a 10% to 20% relative response, would result in fewer "typing responses". Third, sufficient linkage disequilibrium exists between certain alleles encoding DR, DQ and other specificities; this linkage disequilibrium in turn leads to sufficient gene, and thus antigen, sharing between the HTC's and the cells of individuals being tested to provide typing responses. To the extent that HLA alleles, such as those of DP, are not in linkage disequilibrium with the DR-DQ combination of a given haplotype, stimulation of proliferation by products of those DP genes creates "noise", which makes more difficult the assignment of a Dw specificity.

Certain relationships exist between the class II serologically defined (Ia) and T lymphocyte defined (Dw/LD) specificities. One speaks of one specificity as being "supertypic" to another (the latter being called "subtypic" to the first) based on population studies. For instance, DQw1 is generally supertypic to DR1, DR2 and DRw6; individuals positive for DQw1 can be divided into those that are positive for DR1, DR2 etc. Also, any individual typing positively for DR1, DR2 or DRw6 is also likely to carry the specificity DQw1. The DQ Ia and DRw52 and w53 specificities are supertypic to the DR1-DRw14 specificities; in addition, certain of the DR1-DRw14 specificities are supertypic to the various Dw specificities.

The Dw specificities defined with HTCs were first thought by some to be the equivalent of the serologically defined DR antigens but there is now much evidence that one serologically defined DR specificity can be associated with several Dw subtypes as defined with HTC. Although the Dw subtypes are usually referred to as being related to a given DR specificity, they also relate to the supertypic DQ Ia specificities. Thus, DQ and DR product-associated LD determinants contribute to the definition of the Dw specificity. One can appropriately speak of DR4-DQw3 or DR2-DQw1 Dw subtypes in the Caucasian population in which DR4 is generally in linkage disequilibrium with DQw3 and DR2 with DQw1.

At least four different Dw specificities can be defined within DR4-DQw3: Dw4, Dw10, Dw13 (formerly DB3) and Dw14 (formerly LD40); Dw15 (formerly DYT) is a subtype of DR4 but is not associated with DQw3 (Reinsmoen and Bach, 1982). DR2-DQw1 can be split into Dw2, Dw12 and LD-MN2 (Reinsmoen et al., 1985; Bach et al., 1985). Some DR4+ or DR2+ individuals carry "blank" (undefined) Dw specificities.

Information relating to the Dw specificities has recently been reviewed in detail (Bach, 1985). The most important conceptual point is that Dw specificities are really "haplotype designations" representing the constellation of determinants, associated with the various class II products, that make up the Dw specificity. As mentioned earlier, we shall refer to individual determinants, recognized by T lymphocytes and associated with the class II products, as LD determinants.

For many, and perhaps all, haplotypes expressing a given Dw specificity, DR, DQ, and DP products will each contribute LD components. This seems most likely but is proven in only some cases. It is possible, for instance, that with respect to DR and DQ, the DR LD specificities of Dw4 and Dw14 are identical

and the stimulation seen in a primary MLC between DR4-Dw4 and DR4-Dw14 is due to disparity between DQw3 associated LD determinants.

A major goal, then, is to identify LD determinants associated with individual $\alpha\beta$ dimers. One approach to this has been the use of "cloned" T lymphocytes discussed below.

Studies With "Cloned" T Lymphocytes

We have used both cytotoxic T lymphocyte clones and non-cytotoxic clones that proliferate in response to antigen in these studies. It is not clear whether the determinants recognized by the two types of clones are the same or not. Results obtained with the two types of clones, however, were similar except in one regard. Whereas several cytotoxic clones were found that detected determinants associated with the Dw subtype (see below), individual proliferating clones usually had added reactivities. Responding populations of antigen-proliferating "clones" obtained from plating 5 cells per well, however, did show subtype association. Since cytotoxic clones show clearer patterns of inhibition with monoclonal antibodies than do non-cytotoxic, proliferating clones, we show those results, recognizing the above caveat.

We have studied both DR4-Dw4 and DR2-Dw2 haplotypes; the results are similar and we shall illustrate them for DR2-Dw2 (Reinsmoen and Bach, submitted for publication). Following sensitization, clones were tested for their reactivity to a panel of cells previously characterized for DR, DQ and Dw specificities; monoclonal antibodies directed at DR or DQ were also used to attempt to block clonal reactivity. Anti-DP or anti-class I clones were eliminated from study. Two findings of import came from these studies. First, clones were detected that reacted only with cells carrying the sensitizing Dw specificity, i.e. Dw2. These clones did not react with DR2 positive cells carrying other Dw specificities (e.g. DR2-Dw12 or DR2-FJO or DR2-LD5a) nor with DR2 negative cells. These

clones, thus, detected a determinant(s) that had the population distribution of the Dw2 subtype (Table 1). Some of these Dw subtype specific clones could be blocked utilizing anti-DR mAb whereas others were blocked by anti-DQ mAb. It would appear, therefore, that determinants associated with both DR and DQ can be correlated with the Dw subtype designation as defined with HTC's.

Second, in both the DR4-Dw4 and the DR2-Dw2 sensitizations, clones were detected that appeared to recognize a determinant associated with what may be the DR α 2 related DR Ia specificity, i.e. DRw53 for the DR4 situation and a presumed, and as yet unnamed, "DRw51" specificity for DR2. Assignment of the antigen specificity recognized by these clones was made, once again, based on both panel testing and blocking with mAb. For DRw53 associated recognition, we used PL3, an mAb obtained from Knowles, Dupont and their colleagues, who have characterized this antibody (Horibe et al., 1984; Knowles et al., submitted for publication). For the presumed DR2 associated DR α 2 specificity, we used an mAb (Kasahara et al., 1983) which has been studied for its ability to block T cell reactivity (Nishimura et al., 1985), and which we believe may react with a determinant that is encoded by a gene allelic to those encoding DRw52 and DRw53, an interpretation also recently given by Sone et al. (1985).

The above studies with cloned T lymphocytes deal with DR and DQ. With regard to DP, we have suggested that that DP polymorphism may be analogous to a Dw/LD subtype polymorphism such as that which is subtypic to DR4 (Nicklas et al., 1985; Bach, 1985). Recent results that we have obtained suggest complexity of the DP region in addition to that defined as the DPw1-DPw6 segregant series. In studying an mAb derived by Trowbridge and colleagues (Royston et al., 1981), we were able to demonstrate that this mAb reacted with HLA class II molecules other than DR and DQ (Watson et al., 1983; Linner and Bach, 1984). The non-DR, non-DQ reactivity of the mAb, which we referred to as anti-FA, was confirmed in the

International Histocompatibility Workshop, and shown to be very specific to the DP family (Crumpton et al., 1984).

Recently, we have used this mAb to block cloned T lymphocytes reactive with DP region encoded molecules based on mapping studies with HLA deletion mutants. Anti-FA blocks cloned cytotoxic T lymphocytes that appear to detect determinants identical to, or very highly associated with, the relevant DP specificity as defined with primed lymphocyte typing (PLT) reagents (Ohta et al., 1985; Ohta and Bach, submitted). (The caveat regarding the potential difference between determinants seen by non-cytotoxic vs. cytotoxic cells also obtains here.) This is, thus, the first evidence that the anti-FA mAb presumably reacts with the protein dimer that expresses the DPw specificities defined with T cell reagents.

By utilizing blocking with an mAb obtained from Dr. Peter Wernet, Tu39, we have found that certain cytotoxic T lymphocyte clones detect a DP region encoded determinant that is not associated with any known specificity of the PLT-defined DPw segregant series (DPw1-DPw6). We have referred to this determinant as NO1. Clones detecting NO1 are blocked by TU39 and not anti-FA. (Ohta and Bach, submitted).

We have used the anti-FA mAb to study DP region encoded α - and β -chain polymorphism. We have shown extensive β -chain polymorphism that overall appears to show correlation with the DPw type. Exceptions that exist to this correlation between isoelectric focusing of DP β -chains detected with anti-FA and the DPw phenotype (Linner and Bach, 1984) could have two explanations. First, protein polymorphism need not, necessarily, be correlated with determinants recognized by T lymphocytes. Second, some of the polymorphism seen, that does not correlate with the DP phenotype, may reflect polymorphism related to determinants such as NO1.

In summary, then, we have provided evidence that determinants associated with both DR and DQ contribute to subtype designations; further, determinants associated with a presumed DR α β 2 dimer are recognized by cloned T lymphocytes. Further, DPw specificities may represent a polymorphism similar to the Dw/LD subtype polymorphisms of a serologically defined DR specificity, such as DR4. It was with a view to obtaining further information regarding T lymphocyte recognition that the molecular studies, described below, were done.

PROTEIN STUDIES

Molecular Basis for LD/Dw Subtypes

In an attempt to detect a molecular polymorphism at the protein level that correlated with the Dw subtypes, we studied DR4 β chains (Groner et al., 1983, Bach and Watson, 1983). HTC8 from several DR4+ individuals whose cells expressed different Dw subtypes were used in these studies. The radiolabeled DR products were precipitated by a monoclonal antibody directed at DR and analyzed in a two dimensional SDS-IEF gel. We found a very strong correlation between IEF positions of DR4 β chains and their Dw subtype. Four different focusing patterns were found for the five different subtypes studied (β chains of Dw4 and Dw14 cells focused identically); all β chains from cells of different individuals expressing the same Dw subtype focused identically (Figure 1). Similar results were obtained after neuraminidase treatment of DR β chains, eliminating the possibility that charge differences are due to sialic acid moieties (Bach and Watson, 1983). Nepom, Hanson and their colleagues (1983) have obtained similar results.

Digestion of DR β chains with α -chymotrypsin, which cleaves the β chain between the first and second domains to yield two fragments that correspond closely to the protein products of the first and second exons for DR β (Kaufman and Strominger, 1982), revealed that the protein differences resulting in

positional variation in IEF of the different β chains resides in the N-terminal domain of the molecule (Bach and Watson, 1983). Although the most reasonable interpretation of these results is that the protein polymorphism correlates with the presence of determinants recognized by T lymphocytes, it is important to note that despite the very strong correlation of Dw subtypes with positional variation in IEF, protein polymorphism, detected, for example, by IEF, does not necessarily identify the determinant recognized by T cells; further, identity in 2-dimensional gels does not prove protein or LD identity. As shown in figure 2, for instance, Dw4 and Dw14 β chains do differ in their peptide maps following trypsin digestion and analysis on high performance liquid chromatography (HPLC) (23), even though no differences were seen between these proteins in the SDS-IEF system (Bach et al., 1984). Although the majority of studies such as these performed to date have examined the DR product, work from several laboratories has revealed differences in isoelectric focusing between DQ β chains associated with a single DQ Ia specificity (Nepom et al. 1984; Bach et al., 1985). The molecular polymorphism of DR and DQ β chains, which is more extensive than the serologically defined polymorphism, is, thus, probably partly related to class II product associated LD polymorphism.

Recently, Nishimura, Sasasuki and their colleagues (1984) have provided an important addition to these studies. At least two separate DR β chains are revealed by IEF-SDS two-dimensional analysis. These workers have shown that in DR2+ cells, a protein which they refer to as DR β_1 is invariant whereas a β_2 molecule is positioned differently in preparations from Dw2 and Dw12 cells. However, when a DR2-negative responding cell is stimulated by a DR2-positive cell, LD determinants apparently associated with both β_1 and β_2 can stimulate proliferation as demonstrated by mAb blocking experiments. The suggestion is that the differences detected in IEF associated with the β_2 chain are

responsible for the definition of Dw2 and Dw12 as subtype specificities. Further studies will be needed to evaluate whether LD determinants that can be recognized between two individuals who share the same serologically defined DR specificities, but differ in their Dw/LD subtype, can also be associated with what Nishimura et al. refer to as the β_1 chain, a situation that seems quite likely. Individual β_1 and β_2 proteins cannot be related to the genes that are referred to as β_1 and β_2 given our present state of knowledge.

DNA STUDIES

SOUTHERN STUDIES OF Dw SUBTYPES

We undertook a Southern blot analysis of restriction fragment length polymorphism (RFLP) in the Dw subtypes of DR4 and DR2 (Nicklas et al. 1985; Segall et al., 1985). In addition, we were interested in the frequency with which RFLP could be demonstrated as being associated with Dw subtypes within the DR4 or DR2 Ia specificities as compared with RFLP seen between different DR Ia specificities.

Since DR β and DQ β probes are known to cross-hybridize, depending on hybridization conditions, a comparison was made of the restriction fragment patterns obtained with the two probes. Chromosomal DNA was cleaved with 4 different restriction enzymes and run in duplicate on a single gel, then half the blot of this gel was probed with the DR β probe and half with the DQ β probe. In most instances, the darkest bands obtained with one probe were lighter on the half of the blot on which the other probe was used. This suggests that the difference in band intensity reflects preferential hybridization of DR β and DQ β probes with DR β and DQ β -associated fragments respectively; faint bands occur as a result of cross-hybridization. Bands which were faint with both probes may be DP associated fragments. From these

results, it appears that the restriction fragments coding for the different genes can be differentiated by the intensity of hybridization.

Initial experiments were done using the DQ β probe. DNA preparations from eight DR4 HTCs (3 Dw4, 2 Dw10, 2 Dw14, 1 Dw15) were compared after digestion with 10 restriction enzymes (BamHI, BglI, BstEII, EcoRI, HindIII, KpnI, PstI, PvuII, SacI, XbaI). Figure 3 shows a Southern blot of the "DR4 DNAs" cleaved with PstI. HAS-15 (Dw15), Lane 1, shows a fragment of 4.1 kb which is missing from all the other samples while the 12.5 kb fragment present in all other samples is lacking. LS40 (Dw14), Lane 2, appears to be heterozygous for the change seen in HAS-15 as it has both the 4.1 kb and 12.5 kb specific bands but at half intensity.

Differences between the DNA preparation from the 8 DR4 cell lines were only seen with 2 of 9 other enzymes tested. With BglI a 7.2kb fragment which was present as a band of high intensity in 5 of the DNA preparations, was absent from the HAS (Dw15) DNA and present but of lighter intensity (suggesting possible heterozygosity) in JS (Dw4) and LS40 (Dw14). Additionally, following BglI digestion, HAS (Dw15) DNA lacked a 2.4 kb fragment detected in all 7 of the other DNA preparations. Southern blotting analysis of BstEII digested DNA offered the only other detectable difference, a 2.5 kb band which appeared for LS40 (Dw14) DNA, but in none of the other preparations. No differences were seen which were consistent for any Dw type where more than one HTC was studied.

Using the DR β probe, one example of each of four Dw types (BSM-Dw4, LS40-Dw14, HAS-Dw15, AL-10-Dw10) was tested with 12 restriction enzymes (ApaI, BamHI, BglI, BglII, BstEII, EcoRI, HindIII, KpnI, PstI, PvuII, SacI, TaqI) to search for RFLP between Dw subtypes. The only differences were detected using BglI; therefore, a Southern blot was prepared using this enzyme to digest all 8

of the DNA preparations from DR4 cell lines from 4 Dw types (see Figure 3). A 5.8 kb fragment was found in DNA from HAS (Dw15, lane 3), CHOP40 (Dw14, Lane 2) and AL10 (Dw10, lane 5) which was not present in other cells; additionally, the 20 kb band of HAS (Dw15) DNA appears to be of lighter intensity than that of the other preparations.

The initial impetus for this study using Southern blotting was the hope of finding Dw associated DNA polymorphisms in the DR4 cells to complement identification of Dw associated protein polymorphisms initially described from this laboratory (Groner et al., 1983; Bach and Watson, 1983). Although a few differences were found, they were rather rare and there was no obvious correlation of RFLP with Dw type. As this was a somewhat unexpected result, comparisons of different DR Ia types were made to demonstrate that RFLP could indeed be detected by our protocol. As described by others (Paulsen et al., 1984; Cohen et al., 1984), frequent RFLP was detected using every restriction enzyme tested, indicating that DR or DQ Ia associated RFLP is much more extensive than Dw-associated related RFLP (Nicklas et al., 1985).

Our finding of an RFLP difference between DR4-Dw15 (HAS) and other Dw subtypes of DR4 with a DQ β probe is not surprising since DR4-Dw15, unlike the other subtypes of DR4, is not associated with DQw3. The difference in this case may, thus, involve a DQ Ia difference.

We have also studied the DR2 family of subtypes: Dw2, Dw12, (LD-5a, a DR2-associated specificity) related to the DB9 specificity defined in the Eighth International Histocompatibility Workshop. Using a series of DR2+ HTC's, we have found RFLP that appear to be associated with subtype (Segall et al., 1985). For example, an Eco RI fragment of about 4.3 kb, detected with the DR β probe, was found in the Dw12 HTC's but did not appear in either Dw2 or LD-5a. Although the Dw2 and Dw12 cells were DQw1 and the LD-5a cells were

DQw3, the RFLPs detected with the DQ β probe appeared to be related to Dw subtype and not to DQw specificity; for example, a 2.2 kb Eco RI fragment was present only in the Dw2 HTCs and not in Dw12 or LD-5a.

We also tested several heterozygous cells carrying the DR2-associated PLT-defined specificity LD-MN-2 (Bach et al., 1985). By PLT analysis, LD-MN2 is related to the DB9 group, as are the HTCs FJO and AZH (Reinsmoen et al., 1984), although LD-MN2 cells do not give typing responses to the LD-5a or FJO HTCs. LD-5a, LD-MN2 and FJO-AZH can all be distinguished from one another by polymorphism for at least one restriction fragment detected with either a DR β or DQ β probe. In all cases, however, the RFLP differences between subtypes are very much less pronounced than the differences between different DR Ia specificities.

Thus, although RFLP patterns detected by Southern blotting and associated with different DR-DQ Ia specificities are easily shown, it appears more difficult to define RFLP patterns associated with the various cellularly defined subtypes of the serologically defined DR4 and DR2 specificities. This is compared quantitatively in Table 2. Since RFLP sites leading to differences in Southern blots are present not only within exons encoding the various class II products, but also presumably within introns and flanking sequences, a model of D region molecular evolution consistent with these data might suggest the following.

First, there has been sufficient time in evolution for the accumulation of DNA sequence variation in introns and flanking regions during the establishment of the DR-DQ Ia polymorphism. This would result in the RFLP differences noted.

Second, the HLA haplotypes currently found in our population are derived from a very few ancestral HLA haplotypes already carrying (expressing) the DR

and DQ Ia polymorphisms as well as mutations in introns and flanking regions associated with each Ia specificity. The existence of only these very few haplotypes (defined by RFLP) would perhaps be due to an evolutionary "bottleneck(s)" or population crisis. The presence of only one, or a very few, ancestral haplotypes for each Ia specificity with its (their) particular complement of associated mutations, would account for the specific RFLP patterns associated with each DR Ia specificity (Cohen et al., 1984; Andersson et al., 1984).

Third, the Dw specificities that we recognize as subtypes of various DR-DQ Ia specificities are of more recent evolutionary origin, i.e. there has not been time for the accumulation of much DNA sequence variation, including that in introns and flanking regions, associated with each Dw subtype and, thus, there is little Dw subtype-associated RFLP.

Fourth, the organism with a new Dw mutation may have a selective advantage, very possibly immunological, perhaps by being heterozygous or simply by being relatively rare. In the case of selective advantage of mutation, changes in DNA encoding proteins will accumulate more rapidly than changes in DNA leading to restriction site polymorphism, since the latter presumably will not be selectively maintained in the population as long as they do not affect the protein product favorably. Since serologically detectable changes do not seem to have accumulated appreciably more rapidly than restriction site changes, cellular determinants of the DR-DQ molecules may be more strongly favored in evolutionary selection than the serological, a possibility which has important immunologic ramifications.

SEQUENCING OF CLASS II β GENES

Although very few sequences are available for HLA class II β genes (41-x), it seems that there are at least three areas within the first exon

(encoding the N terminal domain of the protein) that appear to be more variable than other stretches of DR β DNA. It is impossible to know which sequence variation may relate to the encoding of LD determinants since sequences available to date have been from cells carrying different DR Ia as well as different Dw/LD specificities.

In an attempt to investigate this question, we have prepared cDNA libraries from DR4 HTC α s that differ with regard to the Dw subtypes that they express (Cairns et al., 1985). Sequences of two DR β cDNAs that we have obtained from a DR4-Dw14 HTC are identical with two exceptions. First, there are thirty additional amino acids encoded by the 3' end of one of these clones (LS1.1.1). Second, the 3' untranslated region of that same clone bears no sequence homology with the second clone (LS5.8.1) or with the 3' untranslated region of any published class II cDNA sequence.

The sequence at the 3' end of LS1.1.1 has been compared to several genomic DR β genes (Larhammar, Peterson, personal communication). It appears that the region encoding the last 61 nucleotides at the 3' end of the coding sequence, and the remaining 3' untranslated region of our clone are approximately 85% homologous with an Alu-1 type repetitive element found 3' to the 6th (last) exon of a DR β pseudogene. The 63 nucleotides immediately 5' to this region of our cDNA clone show nearly 90% homology with the junction between the 5th exon and the 5' end of the intron separating the 5th from the 6th exons of the DR β pseudogenes (Larhammar et al., 1985). This degree of homology between our clone and the DR β pseudogene makes it unlikely that the unusual sequence at the 3' end of our clone is the result of some artifact occurring during the cloning procedure but supports instead the concept that the two clones we have sequenced represent differentially processed transcripts of the same gene.

Surprisingly, the sequences we obtained from our two clones from the DR4-Dw14 HTC were identical to a DR β sequence obtained by Long, Mach et al. (unpublished data) from a cell that we have identified as most probably carrying a DR4-Dw4 haplotype or at least components of a Dw4 haplotype with the exception of three nucleotides which result in two amino acid differences, one a lysine to arginine substitution at position 71, the second at position 86 of the first domain resulting in a glycine to valine substitution. Although at present it is difficult to assign the clones we have sequenced to a particular DR β locus, these results may indicate that major differences recognized by T cells on DR molecules may be conferred by relatively minor changes in the amino acid sequences of these molecules, a result similar to the situation regarding T cell recognition of class I molecules.

SOME COMMENTS

Despite these advances, there are some areas in which there is at least some measure of current difficulty. We discuss just one of these, once again with focus on certain data obtained in our own laboratories.

There is the question of interrelating class II genes, proteins and reactive T cells. Monoclonal antibodies can be used to block reactivity of cloned T cells and thus permit tentative assignment of those T cells to a particular protein, although such blocking studies cannot be interpreted unambiguously (Strassmann et al., 1985; Anichini et al., 1985). Further, the very high degree of homology of different class II β genes, especially within a family, with the non-availability of probes that are specific for different loci of DR, for instance, does not make easy present assignment of DNA sequences to DR β_1 , DR β_2 , or DR β_3 proteins.

The two DR β sequences, determined in our own laboratories and referred to herein (Cairns et al., 1985), are illustrative of these difficulties. In their

coding regions with the exception of the added 30 amino acids in one of them, our sequences are identical to a sequence determined by Long and co-workers (unpublished data) with the exceptions discussed immediately above. Since we were able to determine the phenotype of the cell from which Long obtained his sequence as one probably having a DR4-Dw4 haplotype (or at least components of a Dw4 haplotype), we have suggested that the two amino acids which differ between these sequences result in major differences in the molecule which are recognized by T cells (Cairns et al., in preparation). However, the possibility remains that the clones we have sequenced represent transcripts of a relatively conserved gene which is apparently not recognized by available T lymphocyte typing reagents. Indeed, sequencing of 3 DR β genes at the genomic level in a DR4-Dw4 HTC (Larhammar et al. 1985, and personal communication) has not turned up sequences showing, as compared to our clones or that of Long, minimal differences such as those we have described.

It is to be expected that problems such as these will soon be solved. The armamentarium of techniques available for study of class II molecules including the use of cloned T cells detecting allo- and restricting determinants, monoclonal antibodies defining class II protein products, cDNA constructions of sequenced genes that have been changed by site directed mutagenesis and their subsequent transfections, and others will rapidly allow definition and further understanding of T lymphocyte stimulating determinants.

ACKNOWLEDGEMENTS

We thank Dr. Eric Long for his willingness to compare our DNA sequences with his unpublished sequence; Dr. Dan Larhammar, for his comparison of the 3' untranslated sequence of LSl.1.1 with his unpublished sequences; and Mrs. Nancy Andresen and Connie Greenberg for their editorial help.

REFERENCES

- Amos, D.B. & Bach, F.H.. (1968) Phenotypic expressions of the major histocompatibility locus in man (HL-A): leukocyte antigens and mixed lymphocyte culture reactivity. *J. Exp. Med.* 128:623.
- Andersson, M., Bohme, J., Andersson, G., Moller, E., Thorsby, E., Rask, L., & Peterson, P.A. (1984) Genomic hybridization with class II transplantation antigen cDNA probes as a complementary technique in tissue typing. *Human Immunol.* 11:57.
- Anichini, A., Strassmann, G., & Bach, F.H. (1985) Inhibition of anti-class I cytotoxicity by anti-class II monoclonal antibodies (MCA). II. Blocking of anti-class I CTL clones by anti-DR MCA. *Human Immunol.* (in press).
- Bach, F.H. HLA class II genes/products--the HLA-D region. (1985) *Immunol. Today* (in press).
- Bach, F.H., Albertini, J.F., Amos, D.B., Ceppellini, R. Mattiuz, P.L. & Miggianno, V.C. (1969) Mixed leukocyte culture studies in families with known HL-A genotypes. *Transplant. Proc.* 1:339-341.
- Bach, F.H. & Hirschhorn, K. (1964) Lymphocyte interaction: a potential histocompatibility test in vitro. *Science.* 143:813-814.
- Bach, F.H., Linner, K.M., Choong, S.A., & Groner, J.P. (1984) Molecular polymorphism of DR and DC products. In: Histocompatibility Testing 1984, ed. Albert, E.D., Baur, M.P. Mayr, W.R., Springer-Verlag, (in press).
- Bach, F.H., Reinsmoen, N., & Segall, M. (1983) Definition of HLA antigens with cellular reactants. *Transplant. Proc.* 15:102.
- Bach, F.H., Rich, S., Barbosa, J. & Segall, M. (1985) Insulin dependent diabetes--associated HLA-D region encoded determinants. *Human Immunol.* 12:59.

- Bach, F.H. & Watson, A.J. (1983) Dw/LD associated molecular polymorphism of DR-4 β -chains: Intramolecular localization of polymorphic sites. *J. Immunol.* 131:1622-1625.
- Bain, B., Vas, M.R. & Lowenstein, L. (1964) The development of large immature mononuclear cells in mixed leukocyte cultures. *Blood* 23:108.
- Cairns, S., Dahl, C., Curtsinger, J., Freeman, S., Nelson, P., Alter, B.J. & Bach, F.H. Sequence analysis of HLA-DR β chains: comparison among Dw subtypes of a single serologically defined specificity. *Fed. Proc.*, abstract, (in press).
- Cohen, D., LeGall, I., Marcadet, A., Font, M.-P., Lalouel, J.-M., & Dausset, J. (1984) Clusters of HLA class II β restriction fragments describe allelic series. *Proc. Natl. Acad. Sci.* 81:7870.
- Crumpton, M.J., Bodmer, J.G., Bodmer, W.F., Hayes, J.M., Lindsay, J. & Rudd, C.E. Biochemistry of class II antigens: workshop report. In: Histocompatibility Testing 1984 ed. Albert, E.D., Baur, M.P., Mayr, W.R., Springer-Verlag, in press.
- Groner, J., Watson, A. & Bach, F.H. (1983) Dw/LD related molecular polymorphism of DR4 beta chains. *J. Exp. Med.* 157:1687-1691.
- Gustafsson, K., Wiman, K., Emmoth, E., Larhammar, D., Bohme, J., Hyldig-Nielsen, J.J., Ronne, H., Peterson, P.A. & Rask, L. (1984) Mutations and selection in the generation of class II histocompatibility antigen polymorphism. EMBO 3:1655-1661.
- Horibe, K., Flomenberg, N., Pollack, M.S., Adams, T.E., Dupont, B. & Knowles, R.W. (1984) Biochemical and functional evidence that an MT3 supertypic determinant defined by a monoclonal antibody is carried on the DR molecule on HLA-DR7 cell lines. *J. Immunol.* 133:3195.

- Hurley, C.K., Giles, R.C., Nunez, G., DeMars, R., Nadler, L., Winchester, R., Stastny, P. & Capra, J.D. (1984) Molecular localization of human class II MT2 and MT3 determinants. *J. Exp. Med.* 160:472.
- Kasahara, M., Ogasawara, K., Ikeda, H., Okuyama, T., Ishikawa, N., Takenouchi, T., Wakisaka, A., Kikuchi, Y. & Aizawa, M. (1983) A monoclonal antibody that detects a polymorphic determinant common to HLA-DR1 and 2. *Tissue Antigens.* 21:105.
- Kaufman, J.F., Auffray, C., Korman, A.J., Shackelford, D.A. & Strominger, J. (1984) The class II molecules of the human and murine major histocompatibility complex. *Cell* 36:1-13.
- Kaufman, J.F. & Strominger, J.L. (1982) HLA-DR light chain has a polymorphic N-terminal region and a conserved immunoglobulin-like C-terminal region. *Nature* 297:694.
- Knowles, R.W., Flomenberg, N., Horibe, K., Winchester, R., Radka, S.F. & Dupont, B. Biochemical characterization of the DR beta chains which carry the two distinct HLA-DRw53-related determinants defined by PL3 and 109d6 and the HLA-DR7-related determinant defined by SFR16-DR7. (manuscript submitted).
- Kratzin, H., Yang, C.Y., Gotz, H., Pauly, E., Kolbel, S., Egert, G., Thinnies, F.P., Wernet, P., Altevogt, P. & Hilschmann, N. (1981) Primary structure of class II human histocompatibility antigens. *Hoppe-Seyler's Z. Physiol. Chem.* 362:1665-1669.
- Larhammar, D., Hyldig-Nielsen, J.J., Servenius, B., Andersson, B., Rask, L. & Peterson, P. (1983) Exon-intron organization and complete nucleotide sequence of a human major histocompatibility antigen DC β gene. *Proc. Natl. Acad. Sci. (USA)* 80:7313.

- Larhammar, D., Schenning, L., Gustafsson, K., Wiman, K., Claesson, L., Rask, L. & Peterson, P.A. (1982) Complete amino acid sequence of an HLA-DR antigen-like β chain as predicted from the nucleotide sequence: Similarities with immunoglobulins and HLA-A, -B, and -C antigens. *Proc. Natl. Acad. Sci. (USA)* 79:3687-3691.
- Larhammar, D., Servenius, B., Rask, L. & Peterson, P.A. (1985) Characterization of an HLA-DR β pseudogene. *Proc. Natl. Acad. Sci. USA*, (in press).
- Linner, K.M. & Bach, F.H. (1984) HLA-FA: A non-DR, non-DC HLA class II product. In: Histocompatibility Testing 1984, ed. Albert, E.D., Baur, M.P., Mayr, W.R., Springer-Verlag, in press.
- Long, E.O., Wake, C.T., Gorski, J. & Mach, B. (1983) Complete sequence of an HLA-DR β chain deduced from a cDNA clone and identification of multiple non-allelic DR β chain genes. *EMBO* 2:389.
- Nepom, B.S., Nepom, F.T., Mickelson, E., Antonelli, P. & Hansen, J.A. (1983) Electrophoretic analysis of human HLA-DR antigens from HLA-DR4 homozygous cell lines: correlation between β -chain diversity and HLA-D. *Proc. Natl. Acad. Sci. USA* 80:6962.
- Nepom, G.T., Nepom, B.S., Antonelli, P., Mickelson, E., Sliver J., Goyert, S.M. & Hansen, J.A. (1984) The HLA-DR4 family of haplotypes consists of a series of distinct DR and DS molecules. *J. Exp. Med.* 159:394-404.
- Nicklas, J.N., Noreen, H.N., Segall, M. & Bach, F.H. Southern analysis of DNA polymorphism between Dw subtypes of DR4. *Human Immunol.* (in press).
- Nishimura, Y., Tsukamoto, K., Sone, T., Hirayama, K., Takenouchi, T., Ogasawara, K., Kasahara, M., Wakisaka, A., Aizawa, M. & Sasazuki T. (1984) Molecular and functional analysis of class II molecules characteristic to HLA-Dw2 and Dw12. In: Histocompatibility Testing 1984. ed. Albert, E.D., Baur, M.P., Mayr, W.R., Springer-Verlag (in press).

- Ohta, N., Anichini, A., Reinsmoen, N., Strassmann, G., Wernet, P., & Bach, F.H. (1985) Analysis of human class II antigens by cloned cytolytic T cell reagents: A study using HLA loss mutant lymphoblastoid cell lines and monoclonal antibodies detecting the HLA-DP product(s). Human Immunol. (in press.)
- Ohta, N. & Bach, F.H. (1985) An HLA-DP region encoded determinant not associated with DPw antigens. Submitted.
- Paulsen, G., Rask, L., Peterson, P.A., Bohme, J., Grosse-Wilde, H., Moen, T., & Thorsby, E. (1984) DNA restriction fragment length polymorphism of some 9th workshop homozygous typing cells using a cDNA probe specific for DR β . In: Histocompatibility Testing, 1984. ed. Albert, E.D., Baur, M.P. Mayr, W.R. p. 516-517, Springer-Verlag. (in press).
- Reinsmoen, N.L. & Bach, F.H. (1985) Clonal analysis of HLA-DR and -DQ associated determinants - their contribution to Dw specificities. Submitted.
- Reinsmoen, N.L. & Bach, F.H. (1982) Five HLA-D clusters associated with HLA-DR4. Human Immunol. 4:249-258.
- Reinsmoen, N.L., Layrisse, Z., Betuel, H., & Bach, F.H. (1984) A study of HLA-DR2 associated HLA-Dw/LD specificities. Human Immunol. 11:105.
- Royston, I., Omary, M.B., & Trowbridge, I.S. (1981) Monoclonal antibodies to human T-cell antigen and Ia-like antigen in the characterization of lymphoid leukemia. Transplant. Proc. 13:761.
- Schenning, L., Larhammar, D., Bill, P., Wiman, K., Jonsson, A.K., Rask, L. & Peterson, P.A. (1984) Both α and β chains of HLA-DC class II histocompatibility antigens display extensive polymorphism in their amino-terminal domains. EMBO 3:447-452.

- Segall, M., Noreen, H., Schluender, L. & Bach, F.H.. (1985) DNA restriction fragment polymorphisms correlated with subtypes of DR2. Proceedings of 1985 Miami Winter Symposium. In press.
- Sone, T., Tsukamoto, K., Hirayama, K., Mishimura, Y., Takenouchi, T., Aizawa, M. & Sasazuki, T. A novel class II molecule responsible for the primary MLR between HLA-Dwa and Dw12. J. Immunol. (in press).
- Strassmann, G., Ress, S., Anichini, A., & Bach, F.H. (1985) Inhibition of anti class I cytotoxicity by anti class II monoclonal antibodies (MCA).
I. Blocking of bulk non-DR and non-DQ directed cytotoxic T cells by MCA Against DR, DQ and DP. H. Immunol. (in press.)
- Watson, A.J., DeMars, R., Trowbridge, I.S., & Bach, F.H. (1983) Detection of a novel human class II HLA antigen. Nature (Lond.) 304:358-361.
- Yunis, E.J. & Amos, D.B. (1971) Three closely linked genetic systems relevant to transplantation. Proc. Natl. Acad. Sci. 68:3031-3035.

TABLE I Cytolytic Clones - Anti DR2/Dw2 Priming*

		Targets															
SI	S2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
DR	2,3	2,3	2,2	2,2	2,2	2,2	2,2	6,6	1,1	4,4	4,4	4,4	4,4	3,3	3,3	7,7	5,5
Dw	2,3	2,3	2,2	2,2	12,12	FJO	LD-5a	6,6	1,1	4,4	10,10	14,14	13,13	3,3	3,3	7,7	5,5
DQ	1,2	1,2	1	1	NT	1	3	1	1	3	3	3	NT	NT	2	2	3
DP	4,6	4	4,5	2,5	NT	NT	NT	2,4	3,4	6,(2?)	4	6,3	NT	1,3	3,4	4	4
clone #																	
DR directed																	
3-19	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
3-29	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-

DQ directed

3-17	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
1-17	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-
1-12	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
1-109	+	+	+	+	-	+	+	+	-	+	+	-	-	-	-	+	-

*Clones 3-19, 1-84, 3-91, 3-29, 3-27, 1-109 demonstrated strong proliferative reactivity; clones 3-89, 3-17, and 1-17 demonstrated weaker proliferative reactivity.

+ 's indicate that cytotoxic clones were significantly lytic on the given target.

Table II. RFLP within and between DR specificities

<u>Comparison</u>	<u># Comp</u>	<u>Average Bands/Comparison</u>	
		<u>Different</u>	<u>Same</u>
within DR4-Dw4	6	0	6.64
within DR4-Dw10	4	0.17	7.33
within DR4-Dw14	1	0.25	7.38
within DR4	45	0.14	7.25
within DR2-Dw2	15	0.08	8.68
within DR2-Dw12	1	0	8.75
within DR2-LD-5a	1	0.25	8.50
within DR2	28	0.63	8.12
among DR1 through 7	21	5.33	4.38

FIGURE LEGENDS

Figure 1

Figure 1a demonstrates results of isoelectric focusing (in an SDS-PAGE--IEF system) of DR β chains immunoprecipitated from HTC's expressing the Dw subtypes shown. Dw4 and Dw14 focus identically, Dw15 focuses more basically and Dw10 markedly more acidically. In figure 1b are the same results following treatment with neuraminidase. The differential focusing position in IEF is maintained suggesting that it is the primary protein structure that differs.

Figure 2

Two HTC, one expressing Dw4 (MJ4) and the other Dw14 (CHOP40), were metabolically labelled with ^{14}C and ^3H arginine, respectively. Following labelling, the cell extracts were mixed and handled together. The N terminal fragments (following α -chymotryptic digestion) of DR β -chains immunoprecipitated with L243 were subjected to trypsin digestion and separated on a hydrophobic (HPLC) column. As can be seen, the two chains showed a very high degree of homology. However, each has at least one peptide peak that is not present in the other. MJ4 has a peptide around fraction number 118 that is not present in CHOP40 and CHOP40 has a peptide position 126 that is not present in MJ4.

Figure 3

DNA from eight DR4 LCL's cleaved with restriction enzyme PstI. Lane 1 is a Dw15 (HAS). Lanes 2 and 3 are Dw14's (LS40 and CHOP40 respectively). Lane 4 is empty. Lanes 5 and 6 are Dw10's (2046 and AL10 respectively) and Lanes 7, 8 and 9 are Dw4's (BSM, MJ4 and LL4 respectively).

Figure 4

DNA from the Dw subtypes cleaved with BglI. Lanes 1 and 2 are Dw14's (LS40 and CHOP40 respectively). Lanes 3 is a Dw15 (HAS). Lanes 4 and 5 are Dw10's (2046 and AL10 respectively) and Lanes 6-9 are Dw4's (JS, LL4, MJ4 and BSM respectively).

END

FILMED

12-85

DTIC